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Formation and regeneration of protoplasts in *Sclerotium rolfsii* ATCC 201126

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ABSTRACT

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Aims: Different cultural conditions for forming and reverting protoplasts were systematically studied to establish a rapid and efficient protocol for *Sclerotium rolfsii* ATCC 201126.

Methods and Results: Osmotic stabilizer, lytic enzymes and mycelial age were the main factors influencing protoplast yields. An optimized protocol involving 1-h hydrolysis of 45-h-old mycelium with *Trichoderma harzianum* enzymes in a 1 : 1 (w/w) biomass : enzyme ratio and 0·6 mol l^{-1} $MgSO_4$ as osmotic stabilizer was designed to produce approx. 2×10^9 protoplasts per gram biomass dry weight, with 99% viability. Differences on the lytic activity between batches of commercial enzymes were clearly evidenced. Protoplast release was highly efficient showing no remaining cell wall material as witnessed by fluorescent brightener 28. Up to 26% of purified protoplasts developed into the typical filamentous form after 50 h of incubation on 0·6 mol l^{-1} sucrose agar media.

Conclusions: The methodology herein proposed allowed a rapid, inexpensive and efficient protoplast production. Optimum yields were higher or in the order of that elsewhere reported for other *S. rolfsii* strains and the required lytic time was significantly shorter. Purified protoplasts successfully reverted to the filamentous morphology.

Significance and Impact of the Study: The present research reports the former protocol for the isolation and reversion of protoplasts in *S. rolfsii* ATCC 201126 providing key factors to ensure optimum results. In addition, the described procedure constitutes a starting point for downstream genetic manipulation.

Keywords: filamentous fungi, protoplast formation, protoplast regeneration, *Sclerotium rolfsii*.

INTRODUCTION

The filamentous fungus *Sclerotium rolfsii* ATCC 201126 has been extensively studied over the last years by virtue of its ability to excrete large amounts of β-1,3-β-1,6-D-glucan or 'scleroglucan' (Fariña *et al.* 1998). This exopolysaccharide exhibits interesting rheological properties for different industrial areas (Fariña *et al.* 2001) and it has been recently included as one of the most potent biological response modifiers (Pretus *et al.* 1991).

The isolation of fungal protoplasts by means of using lytic enzymes and hypertonic media to provide osmotic support is a well-known technique to obtain cells completely deprived of cell wall. This methodology showed to be suitable for the isolation of organelles, preparation of cell-free extracts and diverse genetic purposes (Hamlyn *et al.* 1981). Protoplasts represent an appropriate source for intact DNA either for electrophoretic karyotype or transformation (Gold *et al.* 1983) and also, for mutagenesis protocols towards strain improvement (Kelkar *et al.* 1990).

Whereas well-established protocols are nowadays available for yeasts, the development of these techniques has not gone smoothly in the case of filamentous fungi. One of the main obstacles with the latter has been the difficulty to find the optimal lytic enzyme, mostly when fluctuations between different batches are detected (Peberdy 1980).

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Although protoplast formation in *S. rolfsii* has been reported before (Deshpande *et al.* 1987; Kelkar *et al.* 1990), any existing protocol should be assessed for each strain under study as emphasized by the significant differences found between protocols, protoplast yields and regeneration rates for two different *S. rolfsii* strains (Deshpande *et al.* 1987; Kelkar *et al.* 1990). Likewise, among the objectives of the present work, the improvement of the available techniques in terms of efficiency, simplicity, required time or costs was further pursued.

Therefore, as the genetic characteristics of *S. rolfsii* ATCC 201126 have not been studied yet, the development of a reliable method for the straightforward and efficient preparation of protoplasts and for their reversion to the filamentous morphology is considered a crucial step. A range of factors which usually influence protoplast release such as culture medium, pretreatment of mycelium, osmotic stabilizers, nature and concentration of lytic enzymes, biomass : enzyme ratio, incubation time and culture age (Deed and Seviour 1989; Kelkar *et al.* 1990) were systematically assessed to define a final protocol for the optimal production and regeneration of protoplasts in this fungus.

MATERIALS AND METHODS

Organism and maintenance

Sclerotium rolfsii ATCC 201126, an isolated strain from rotten red pepper, was preserved as sclerotia in distilled water according to the protocol previously proposed (Fariña *et al.* 1996a).

Culture media

Mycelium growth media. (i) Czapek with ammonium ($CzNH_4$), in g l^{-1}: sucrose, 30; $(NH_4)_2SO_4$, 2·31; yeast extract, 1; K_2HPO_4, 1; $MgSO_4{\cdot}7H_2O$, 0·5; KCl, 0·5; $FeSO_4{\cdot}7H_2O$, 0·01; pH 4·5. (ii) Complex Yeast Medium (CYM), in g l^{-1}: glucose, 20; peptone, 2; yeast extract, 2; K_2HPO_4, 1·31; KH_2PO_4, 0·46; $MgSO_4{\cdot}7H_2O$, 1; pH 4·5. (iii) Deterring Optimized (DOPT) medium, in g l^{-1}: sucrose, 20; $(NH_4)_2SO_4$, 1·75; yeast extract, 1; $K_2HPO_4{\cdot}3H_2O$, 2; $MgSO_4{\cdot}7H_2O$, 0·5; KCl, 0·5; citric acid·H_2O, 0·7; $FeSO_4{\cdot}7H_2O$, 0·05; pH 4·5.

Protoplast regeneration media. (i) Yeast Nitrogen Base (YNB; Difco Laboratories, Detroit, MI, USA), 10 g l^{-1}. (ii) ME, in g l^{-1}: glucose, 20; malt extract, 20; peptone, 1. (iii) MR, in g l^{-1}: sucrose, 20; $(NH_4)_2SO_4$, 2·3; K_2HPO_4, 1; $MgSO_4{\cdot}7H_2O$, 0·5; vitamins (in μg l^{-1}: biotin, 2; Ca-pantothenate, 400; folic acid, 2; inositol, 2000; niacin, 400; *p*-aminobenzoic acid, 200; pyridoxine·HCl, 400; riboflavin, 200; thiamine·HCl, 400) and trace elements (in μg l^{-1}: boric acid, 500; $CuSO_4$, 40; KI, 100; $FeCl_3$, 200; $MnSO_4$, 400; Na_2MoO_4, 200; $ZnSO_4$, 400). (iv) CYM, see above. (v) SYE, in g l^{-1}: sucrose, 100; yeast extract, 10; pH 6·0. (vi) MGY, in g l^{-1}: glucose, 10; yeast extract, 1; malt extract, 1; pH 5·5. All regeneration media were supplemented with different osmotic stabilizers and appropriately solidified with agar or gelatine as indicated (see Results; Table 2).

Cultivation conditions

Mycelium was obtained by germination of water-preserved sclerotia on malt Czapek agar plates incubated at 30°C as previously described (Fariña *et al.* 1996a). Then, 50 ml of liquid medium ($CzNH_4$, CYM or DOPT, see Results) placed in a 250-ml Erlenmeyer flask, inoculated with five mycelium-covered agar discs (approx. 5 mm diameter) removed from a 2-day-old, malt Czapek-grown culture, were aseptically homogenized with an ATMA hand blender (LM855 model, ATMA, Rep. Pop. China) three times at low speed for 10 s and incubated afterwards at 30°C on an orbital shaker at 250 rev min^{-1} for 45 h unless otherwise stated.

Protoplast formation from fungal mycelium

Mycelium from liquid cultures was harvested by centrifugation at 16 300 *g* (20 min, 5°C). The pellet was washed twice with distilled water and once with 0·2 mol l^{-1} citrate-phosphate buffer (CPB) pH 5·8 and then, it was resuspended in the original volume (50 ml) with the same buffer. Biomass dry weight (BDW) was determined by drying the washed mycelium at 105°C to constant weight. At least triplicate determinations were made in each case.

When sulphydryl agents were tested, mycelium was resuspended in a pretreatment solution containing 0·01 mol l^{-1} dithiothreitol (DTT) in 0·2 mol l^{-1} CPB pH 7·3. After 20 min of incubation, mycelium was recovered by centrifugation and washed three times with 0·2 mol l^{-1} CPB, pH 5·8. Homogenization of mycelium before protoplasting, when assessed, was carried out with a blender under aseptic conditions as described above.

Protoplasts were prepared by incubating 15 mg BDW in 5 ml of 0·2 mol l^{-1} CPB pH 5·8 (to obtain about 3 mg BDW per ml) containing the osmotic stabilizer and lytic enzyme, both at appropriate concentrations (see later, Results). Lytic enzymes from *Trichoderma harzianum*, *Rhizoctonia solani* and *Cytophaga* species were purchased from Sigma Chemical Co. (St Louis, MO, USA); lytic enzymes from *suc* d'*Helix pomatia* (Helicase) from Reactifs I.B.F. (Garenne, France) and Novozym 234 from Novo Nordisk A/S (Bagsvaerd, Denmark). Analytical grade osmotic stabilizers (Sigma) were used.

The reaction was carried out in 25-ml Erlenmeyer flasks, shaken at 160 rev min^{-1} and incubated at 30°C. The

criterion for defining protoplasts was the absence of cell wall in conjunction with the osmotic sensitivity of spherical cells when deprived of osmotic stabilizer. Protoplast yield was estimated by counting with an improved Neuebauer-type haematocytometer counting chamber and results were expressed as mean values of at least triplicate determinations from independent cultures. The statistical significance of differences between mean values was assessed using the ANOVA and Tukey–Kramer Multiple Comparisons tests (Miller and Miller 1988).

Viable protoplasts were evidenced by Trypan blue dye (1 mg ml^{-1}) exclusion and the osmotic sensitivity was demonstrated by their rapid lysis after dilution in water (Picataggio *et al.* 1983).

Cell wall staining

Suspensions of mycelium and/or protoplasts were treated with fluorescent brightener 28 (Sigma) at a final concentration of 1 μg ml^{-1} during 45 min. Fluorescence of samples was microscopically examined to detect the presence of cell wall material.

Protoplast regeneration

After digestion, protoplasts were harvested from the reaction mixture and washed twice using 1·2 mol l^{-1} sorbitol as osmoticum in 0·2 mol l^{-1} CPB pH 5·8; centrifugations were carried out at 960 *g* (15 min, 5°C). Protoplast pellets were resuspended in 2 ml of the same washing solution before plating for regeneration.

Broth regeneration. After appropriate dilution, 100–200 μl of the resuspended pellet containing 100 to 200 protoplasts was poured into 50 ml of regeneration medium, in 250-ml Erlenmeyer flasks, and incubated at 30°C in an orbital shaker at 100 rev min^{-1} for 10 days.

Solid medium regeneration. Similarly, an aliquot containing 100 to 200 protoplasts was suspended in molten (45°C) regeneration medium, plated on Petri dishes and incubated at 30°C (agar plates) or 25°C (gelatine plates) for 10 days. Regeneration frequencies were estimated by comparing the direct microscopic counts of viable protoplasts against the number of colonies grown on the regeneration plates.

RESULTS

Culture medium

The lytic conditions preliminarily chosen were 0·6 mol l^{-1} $MgSO_4$ as osmotic stabilizer, 1 : 1 (w/w) biomass : enzyme ratio, lytic enzymes from *T. harzianum* and 45-h-old mycelium. After 90 min of incubation, the protoplast yields achieved with mycelium harvested from $CzNH_4$, CYM and DOPT culture media were $2{\cdot}5 \pm 0{\cdot}4 \times 10^9$, $1{\cdot}9 \pm 0{\cdot}3 \times 10^9$ and $1{\cdot}7 \pm 0{\cdot}5 \times 10^9$ protoplasts per gram BDW, respectively. Nevertheless, the differences in yield were not statistically significant.

Osmotic stabilizer

The efficiency to support protoplasts following cell wall removal under the conditions described above was evaluated for six different osmotic stabilizers. Even when protoplasts were observed in all cases, the highest yields were found when 0·6 mol l^{-1} $MgSO_4$ was used (Table 1a). The influence of the osmotic stabilizer was extremely significant ($F = 50{\cdot}495$; $P < 0{\cdot}0001$) and, on the basis of these data, 0·6 mol l^{-1} $MgSO_4$ was selected for subsequent experiments. When comparing protoplast yields obtained in 0·7 mol l^{-1} NaCl, 0·7 mol l^{-1} KCl, 0·6 mol l^{-1} mannitol, 1·0 mol l^{-1} sorbitol and 0·6 mol l^{-1} sucrose, no significant differences were found in between.

According to the osmotic stabilizer used, some morphological differences were observed. By using 0·6 mol l^{-1} $MgSO_4$, protoplasts exhibited a slightly granular appearance in association with large vacuoles (1–3 per protoplast). When osmotic stabilizers different from $MgSO_4$ were tested, protoplasts were smaller and the number of visible vacuoles was significantly decreased or lacked.

Table 1 Influence of osmotic stabilizer on protoplast formation in *S. rolfsii*

	No. of protoplast yield per gram BDW*
(a) Nature (mol l^{-1})	
$MgSO_4$ (0·6)	$2{\cdot}6 \pm 0{\cdot}3 \times 10^9$
NaCl (0·7)	$4{\cdot}5 \pm 1{\cdot}0 \times 10^8$
KCl (0·7)	$2{\cdot}7 \pm 0{\cdot}8 \times 10^8$
Mannitol (0·6)	$3{\cdot}7 \pm 0{\cdot}7 \times 10^8$
D-Sorbitol (1·0)	$1{\cdot}7 \pm 0{\cdot}7 \times 10^8$
Sucrose (0·6)	$2{\cdot}0 \pm 0{\cdot}6 \times 10^8$
(b) $MgSO_4$ conc. (mol l^{-1})	
0·4	$1{\cdot}3 \pm 0{\cdot}2 \times 10^9$
0·6	$2{\cdot}0 \pm 0{\cdot}3 \times 10^9$
0·8	$2{\cdot}0 \pm 0{\cdot}2 \times 10^9$
1·0	$1{\cdot}7 \pm 0{\cdot}2 \times 10^9$
1·2	$1{\cdot}1 \pm 0{\cdot}4 \times 10^9$

Lytic conditions: 45-h-old mycelium harvested from $CzNH_4$, 1 : 1 (w/w) biomass : enzyme ratio, lytic enzymes from *T. harzianum*, 30°C, 160 rev min^{-1}, 90 min.

*Reported as the mean ± S.E.M. for three independent replicates.

The results using $MgSO_4$ over a range of 0·4–1·2 mol l^{-1} showed no statistically significant differences on protoplast yields (Table 1b). The increase in osmotic stabilizer concentration reasonably determined a protoplast size reduction; protoplasts in the presence of 0·4 mol l^{-1} $MgSO_4$ were bigger than with 0·6 mol l^{-1} $MgSO_4$ as well as extremely fragile. For that reason and as higher $MgSO_4$ concentrations did not improve protoplast numbers, 0·6 mol l^{-1} $MgSO_4$ was the concentration adopted for further experiments.

Lytic enzyme

The major cell wall components of *S. rolfsii*, chitin and glucan (Chet and Henis 1967; Kelkar *et al.* 1990), might be hydrolysed by different commercial enzyme preparations, either as its main or side activities (Hamlyn *et al.* 1981). In practice, the effectiveness of different commercial enzymes on protoplast formation in *S. rolfsii* was investigated. Comparison involved the use of lytic enzymes from the snail *H. pomatia* and other enzymes from microbial origin such as *T. harzianum*, *R. solani*, *Cytophaga* sp. and Novozym 234.

All enzymes were evaluated in a 1 : 1 (w/w) ratio with respect to the BDW. Only when using lytic enzymes from *T. harzianum* and Novozym 234, protoplast formation was observed, with yields of $2{\cdot}4 \pm 0{\cdot}3 \times 10^9$ and $7{\cdot}0 \pm 0{\cdot}4 \times 10^8$ protoplasts per gram BDW, respectively. The difference was statistically significant ($P = 0{\cdot}0303$) so that lytic enzymes from *T. harzianum* were used in following experiments. In both cases, almost the total protoplast yield was reached within the hour of treatment.

Incubation time

Along with the enzymatic removal of cell wall, the maximum protoplast yields were achieved after 60 min of incubation (Fig. 1) and accordingly, this time was considered optimal for protoplast release in all subsequent studies. Micrographs showed that mycelium was first subdivided into small irregular fragments and there was some degree of cytoplasmic shrinkage at the beginning of incubation. Then, the fragments were transformed into distorted cells, and at the end of the incubation, almost all the cells were converted into protoplasts (Fig. 2a–c).

At early stages, protoplasts were mostly emerging from the hyphal tips but, as cell wall degradation progressed, protoplasts emerged from other regions in addition to the tips. Eventually, complete or partially formed protoplasts were connected by threads of residual material, which could be entirely disrupted by shaking thus releasing free protoplasts. The existence of varying size protoplasts at early stages might be related to their release from different parts of the hypha, and the release pattern is likely associated to the enzyme in use and its mode of action (Yamada *et al.* 1983; Ishikawa and Oishi 1985; Deed and Seviour 1989).

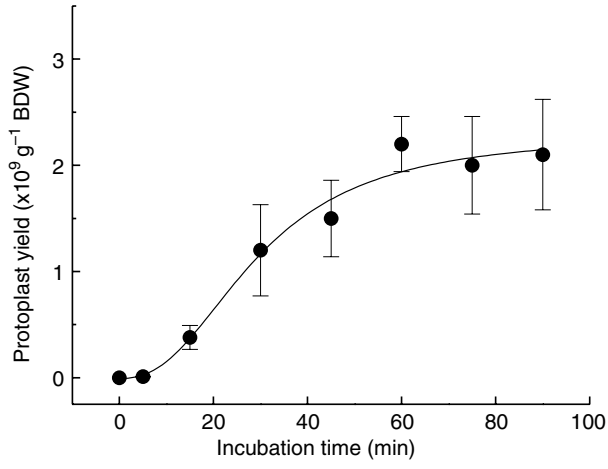

Fig. 1 Kinetic of protoplast formation in *S. rolfsii*. Lytic conditions: 45-h-old mycelium harvested from $CzNH_4$, 0·6 mol l^{-1} $MgSO_4$, 1 : 1 (w/w) biomass : enzyme ratio, lytic enzymes from *T. harzianum*, 30°C, 160 rev min^{-1}. Bar markers represent S.E.M. from mean values of three independent experiments

Biomass : enzyme ratio

It has been previously emphasized that an increase in biomass into the lytic mixture does not automatically yield higher numbers of protoplasts because it depends on the fact that the enzyme does not become limiting, on the organism, and on the lytic conditions. Thus, the optimal biomass : enzyme ratio needs to be investigated for each individual fungal strain (Curragh *et al.* 1992).

When the biomass : enzyme ratio varied between 3 : 1 and 1 : 3 (w/w) under the conditions previously selected, protoplast numbers ranged from $1{\cdot}8 \pm 0{\cdot}5$ to $1{\cdot}1 \pm 0{\cdot}2 \times 10^9$ protoplasts per gram BDW, and the differences were not significant. At 1 : 1 (w/w) biomass : enzyme ratio, even at the earlier stages, protoplasts exhibited a well rounded-off cytoplasm and retained no vestiges of the original cell wall as witnessed by wall polymer-specific fluorescent labels. Their treatment with 2 g l^{-1} SDS led to a rapid lysis and no colonies were thereafter detected on regeneration agar thus confirming the total cell wall removal.

However, as biomass proportion was augmented (e.g. for 2 : 1 and 3 : 1 ratios) protoplasts of different dimensions with remaining cell wall material and, in many cases, incomplete release from hyphae were obtained. Conversely, when the enzyme was increased (e.g. for 1 : 2 and 1 : 3 ratios), smaller protoplasts with irregular edges along with a dirty background of cell debris were observed. Based on these morphological features, a 1 : 1 (w/w) biomass : enzyme ratio was selected for following tests.

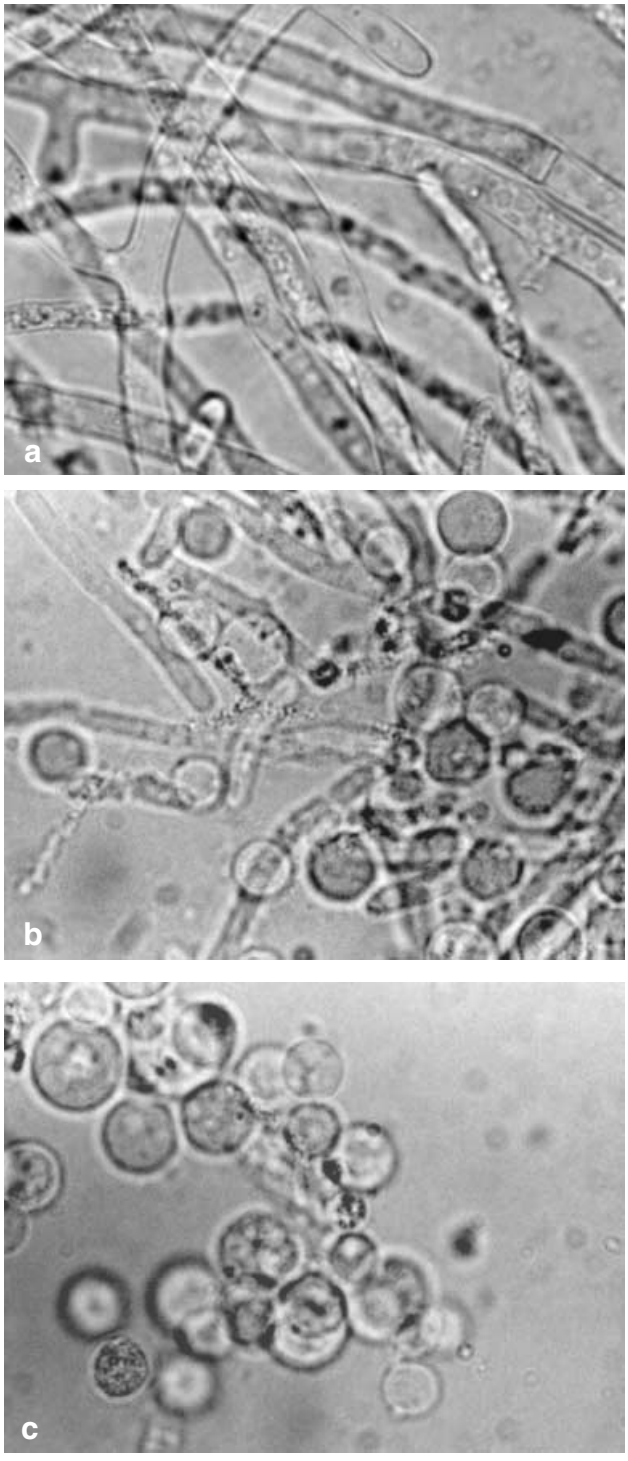

Fig. 2 Morphological changes during *S. rolfsii* protoplast formation under optimized lytic conditions (as in Fig. 1). Micrographs were taken at 1000× magnification after 0 min (a), 15 min (b) and 60 min (c) of incubation

Lytic enzyme batch variations

Considering that batch variations could be a critical factor determining erratic protoplast yields (Picataggio *et al.* 1983), and particularly in view of the fact that the components in lytic systems are frequently side activities, further experiments were included. Namely, this comparison involved different lots of commercially available preparations from *T. harzianum*.

Despite the enzyme spectrum in the tested preparations was quite similar (containing cellulase, protease and chitinase activities), differences on the lytic activity were manifest. For the same concentration (3 mg ml^{-1}), three different batches led to protoplast yields of $2{\cdot}6 \pm 0{\cdot}1 \times 10^9$, $1{\cdot}5 \pm 0{\cdot}3 \times 10^9$ and $2{\cdot}6 \pm 0{\cdot}3 \times 10^8$ protoplasts per gram BDW, and the differences were extremely significant ($F = 60{\cdot}752$; $P < 0{\cdot}0001$).

When the lower yields obtained were tried to be improved, it required a suitable increase in enzyme concentration whose magnitude depended on the batch tested. Once the optimal concentration was found, the kinetic for protoplast release was quite similar to that shown in Fig. 1. Nevertheless, special care should be taken to select the appropriate enzyme concentration as a surplus might lead to a significant decrease in yield (data not shown).

Pretreatment of mycelium

In order to increase or facilitate protoplast release in yeasts, sulphydryl agents have been extensively used in conjunction with lytic preparations. Frequently, either DTT or β-mercaptoethanol was employed to reduce the disulphide bonds present in yeast cell walls (Hamlyn *et al.* 1981; Kavanagh and Whittaker 1996).

Under the optimal lytic conditions established, preincubation with 0·01 mol l^{-1} DTT did not significantly improve yields ($1{\cdot}1 \pm 0{\cdot}1 \times 10^9$ protoplasts per gram BDW) and protoplasts were small with visible vacuoles. Similar results were found in other fungi (Hamlyn *et al.* 1981; Kelkar *et al.* 1990).

As maximizing the number of growing tips for increasing protoplast formation has been previously reported (Curragh *et al.* 1992) and taking into account that *S. rolfsii* tends to form mycelial clumps, the homogenization of mycelium prior to enzyme digestion was also intended as a pretreatment. Unsuccessfully, it led to a significant decrease in yields ($6{\cdot}6 \pm 1{\cdot}1 \times 10^8$ protoplasts per gram BDW) and also, a considerable reduction in protoplast size and number of vacuoles was found.

Culture age

According to the literature, the main factor determining protoplast yields is the physiological status of the

organism at the time of protoplasting; cells at the exponential phase of growth would be the most susceptible (Peberdy 1980). Consequently, we evaluated the efficiency of protoplast production with mycelium of different culture ages, adjusted to equivalent mycelial concentrations.

The best results were obtained with young and actively growing mycelium, which corresponded to 45-h-old cultures (Fig. 3). The use of younger mycelium yielded very small protoplasts and large amounts of cell debris. Starting with mycelium older than 45 h resulted in a large decrease of protoplast yields, in association with a considerable proportion of granular residual hyphae and cell debris. The influence of culture age was statistically significant ($F = 6{\cdot}500$; $P = 0{\cdot}0132$).

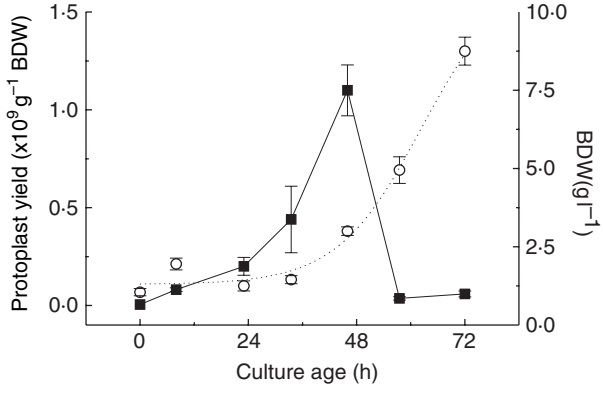

Fig. 3 Influence of culture age on protoplast release in *S. rolfsii*. Lytic conditions: mycelium harvested at different times from $CzNH_4$, 0·6 mol l^{-1} $MgSO_4$, 1 : 1 (w/w) biomass : enzyme ratio, lytic enzymes from *T. harzianum*, 30°C, 160 rev min^{-1}, 60 min. Bar markers represent S.E.M. from mean values of three independent experiments. (○) Growth expressed as biomass dry weight, (■) protoplast yield

Protoplast regeneration

One of the most important features of protoplasts as genetic tools is their reversion to the mycelial form, which constitutes a prerequisite. This event requires hypertonic culture media where cell wall regeneration takes place, and allows the organism to revert to its normal morphology. The levels of regeneration in filamentous fungi can fluctuate significantly and very low regeneration rates are expectable (e.g. ranging from 0·3 to 4·9%) (Peberdy 1980; Curragh *et al.* 1992).

Prior to regeneration tests, protoplast purification was consistently carried out with 1·2 mol l^{-1} sorbitol and centrifugation at 960 *g* (15 min, 5°C), which led to a protoplast recovery around 89%. Particular attention should be paid at this step as preliminary tests using washing buffer with 0·6 mol l^{-1} $MgSO_4$ and centrifugation at 350 *g* (15 min, 5°C) gave significantly lower yields (final pellet represented *ca* 34% of the initial value).

In the present work, protoplasts regenerated cell wall under different cultural conditions (Table 2). Usually, after

Table 2 Influence of culture medium and osmotic stabilizers on protoplast regeneration in *S. rolfsii*

Culture medium*	Osmotic stabilizer (mol l^{-1})	Solidifying agent (g l^{-1})	pH	Regeneration (%)†
YNB-sorbose	L-sorbose (0·6)	Agar (30)	4·8	0
ME-sorbitol	D-sorbitol (0·5) + agar (overlay) (5 g l^{-1})	Agar (support) (20)		0
MR-Mg	$MgSO_4$ (0·6)	Agar (15)	4·5	Culture medium precipitation
MR-sucrose	Sucrose (0·6)	Agar (15)	4·5	0
CYM-Mg	$MgSO_4$ (0·6)	Gelatine (150)	4·5	9 ± 1
CYM-sorbose	L-sorbose (0·6)	Gelatine (150)	4·5	4 ± 1
CYM-sucrose	Sucrose (0·6)	Gelatine (150)	4·5	12 ± 1
SYE-Mg	$MgSO_4$ (0·6)	Agar (30)	6·0	0
MGY-sorbitol	D-sorbitol (1·0)	Agar (20)	5·5	10 ± 2
MGY-sucrose	Sucrose (0·6)	Agar (20)	5·5	26 ± 8
MGY-KCl	KCl (0·7)	Agar (20)	5·5	7 ± 2
MGY-NaCl	NaCl (0·7)	Agar (20)	5·5	17 ± 6

Lytic conditions: 45-h-old mycelium harvested from $CzNH_4$, 1 : 1 (w/w) biomass : enzyme ratio, 0·6 mol l^{-1} $MgSO_4$, lytic enzymes from *T. harzianum*, 30°C, 160 rev min^{-1}, 60 min.
*For medium composition, see Materials and methods.
†Regeneration frequency = (no. of regenerating colonies/no. of viable protoplasts inoculated) × 100, reported as the mean ± S.E.M. for five independent replicates. Osmotically shocked controls and a 99% Trypan blue-tested protoplast viability were also considered for calculations.

50 h of incubation, protoplasts developed into incipient colonies observable with the naked eye. To confirm the typical morphology and nonstop growth, final results were read after 10 days of cultivation. Colonies originated from regenerating protoplasts exhibited sclerotia formation and colony radial growth rate (K_r) similar to the parent strain (Fariña *et al.* 1996b).

The results indicated no plain correlation according to the osmoticum, the solidifying agent or the culture medium used so as to independently promote regeneration. The differences on regeneration rates were extremely significant ($F = 6{\cdot}950$ $P = 0{\cdot}0002$). The highest frequencies were attained when MGY agar medium was supplemented with 0·6 mol l^{-1} sucrose (26%) and 0·7 mol l^{-1} NaCl (17%). There were striking fluctuations between the results depending on the support culture medium used. If 0·6 mol l^{-1} sucrose was added to CYM, then the regeneration values were almost twofold decreased, while on MR medium no regeneration was detected (Table 2).

In the majority of yeast protoplasts, cell wall regeneration takes place in the presence of solidified media, either by means of the addition of agar or gelatine or thickened with polyethylene glycol; regeneration in osmotically stabilized liquid media is exceptional. In the former case, the barrier around the protoplast provided by solidifying agents has been considered responsible of preventing loss of cell wall components and facilitating their accumulation on the protoplast surface (Kavanagh and Whittaker 1996).

Our results further evidenced regeneration in diverse liquid media: CYM + 0·6 mol l^{-1} $MgSO_4$, CYM + 0·6 mol l^{-1} sorbose and CYM + 0·6 mol l^{-1} sucrose. Even when growth was obtained in all cases, differences in the growth pattern were noticed. In the presence of $MgSO_4$, pellets of small size were formed (75–80 pellets per 50 ml) providing a more homogeneous pattern. In contrast, a tendency to develop large clumps was observed in the other media.

DISCUSSION

To gain a greater insight into the genetic characteristics of *S. rolfsii* ATCC 201126 which have not been investigated so far, and considering the biotechnological potential of the strain, the optimization of protoplast production and reversion was undertaken as a first step. It is already known that protoplast formation often constitutes the starting point for downstream genetic manipulation. However, protoplast technology is frequently problematic and less reproducible, particularly for filamentous fungi, thereby emphasizing the necessity to determine the key factors leading to successful results.

Among the aspects under consideration, osmotic stabilizer, lytic enzyme and mycelial age were those which significantly influenced protoplast isolation. The highest protoplast yields were reached by using 0·6 mol l^{-1} $MgSO_4$ and the presence of vacuoles under these conditions constituted a well-documented morphological feature (Deed and Seviour 1990). Similar results were previously reported for other strain of *S. rolfsii* (Deshpande *et al.* 1987) and for *S. glucanicum* BCAE M16 (Deed and Seviour 1989). Differently, Kelkar *et al.* (1990) achieved similar protoplast numbers for *S. rolfsii* NCIM 1084 but using 0·6 mol l^{-1} KCl instead.

The use of $MgSO_4$ as osmotic stabilizer has also been found more effective for protoplast isolation in other filamentous fungi (Deshpande *et al.* 1987; Deed and Seviour 1990; Curragh *et al.* 1992). The beneficial effect of Mg^{2+} for preventing lipid release from the plasmatic membrane (Okanishi *et al.* 1974) or some influence derived from the buffer viscosity have been speculated (Kavanagh and Whittaker 1991). It is important to point out that the selection of the optimal stabilizer would be closely related to the lytic enzyme in use (Kottutz and Rapp 1990). In this case, 0·6 mol l^{-1} $MgSO_4$ was found optimal when used in conjunction with *T. harzianum* enzymes.

From the lytic enzymes herein evaluated, only *T. harzianum* and Novozym 234 were effective. This fact would presumably reflect differences on the key enzyme activities involved in cell wall degradation (Hamlyn *et al.* 1981). The best performance of *T. harzianum* enzymes was in compliance with the α-glucanase, β-glucanase and chitinase activities reported in this fungus, as they would be able to digest the major cell wall components in *S. rolfsii* (Peberdy 1980; Kelkar *et al.* 1990; Kavanagh and Whittaker 1996).

The use of *T. harzianum* enzymes was the most advantageous and yields were higher or similar to those elsewhere reported for other *S. rolfsii* and *S. glucanicum* strains (Deshpande *et al.* 1987; Deed and Seviour 1989; Kelkar *et al.* 1990; Kottutz and Rapp 1990). However, by using this lytic system the period required for protoplast release (60 min) resulted markedly lower than that reported for the same and other related species (ranging from 2 to 24 h) (Deshpande *et al.* 1987; Deed and Seviour 1989; Kelkar *et al.* 1990; Kottutz and Rapp 1990). Furthermore, and contrasting with the literature (Peberdy 1980; Hamlyn *et al.* 1981), the effectiveness of using only one type of commercial enzyme was demonstrated.

As formerly mentioned, different commercial lytic preparations can exhibit a similar profile of side enzymatic activities. However, the variation in protein content that regularly occurs between batches might explain whole lytic activity fluctuations. Consequently, this aspect requires special attention and highlights the importance to assess the lytic activity for the system in use. This factual matter should alert for future investigations.

Mycelial age strongly influenced protoplast yields as previously reported for other *S. rolfsii* strains (Deshpande *et al.* 1987; Kelkar *et al.* 1990). This fact would be related to the changes in the hyphal wall, which passing from the exponential phase of growth becomes more resistant to enzyme degradation. Likewise, it might be also associated to the high number of growing tips when young and actively growing cultures are used for protoplast production (Picataggio *et al.* 1983; Kottutz and Rapp 1990; Curragh *et al.* 1992; Vázquez and Figueroa 1996).

With regard to the culture medium, its influence on protoplast yields has been occasionally reported and changes in the composition or ultrastructure of hyphal wall were hypothesized (Peberdy 1980; Yamada *et al.* 1983). In the present case, apparent fluctuations according to the culture medium used were not statistically significant.

Finally, protoplasts were assessed for their ability to revert to the filamentous morphology. The preferential regeneration with 0·6 mol l^{-1} sucrose instead of the inorganic stabilizer used for protoplast isolation (0·6 mol l^{-1} $MgSO_4$) was not unexpected as possible inhibition of cell wall enzymes has been speculated earlier (Deed and Seviour 1990). The best regeneration frequencies achieved (26%) were higher than those elsewhere reported for *S. glucanicum* BCAE M16 (ranging between 2·5 and 6·8%) (Deed and Seviour 1989) and for *S. rolfsii* isolated from peanut (0·3–0·6%) (Deshpande *et al.* 1987), and similar to the ones obtained for floating protoplasts from *S. glucanicum* NRRL 3006 (24%) (Kottutz and Rapp 1990).

At the time of comparing regeneration rates it should be stressed that values emerging from different protocols, for instance, when the percentage of regeneration is estimated from microscopically examined slides and not from colonies observed on plates, might exhibit a significant divergence (Kelkar *et al.* 1990). The inability of microscopically detected germ tube-forming protoplasts to develop into a true colony afterwards may consequently derive in a reduced regeneration number.

Many hypotheses have been postulated to explain low regeneration values. The clumping of contiguous protoplasts after centrifugation (Deed and Seviour 1989), high proteolytic activities of enzyme preparations, prolonged cell wall exposure (Hamlyn *et al.* 1981), differences in the stability of protoplasts of varying phenotypes (Gold *et al.* 1983) and cultivation conditions (Keller *et al.* 1983) have been shown to notoriously affect regeneration.

Knowledge is still fragmentary on nuclear behaviour of filamentous fungi protoplasts (Peberdy 1980) and for instance, in the case of *S. glucanicum* BCAE M16 the nuclear complement varied between 0 and 5 (Deed and Seviour 1989). The need to convert to uninucleate forms before reverting (Deed and Seviour 1989) and the presence of a large number of enucleate protoplasts (Magae *et al.* 1985) have also been speculated to explain lowered regeneration.

Protoplast production achieving similar yields to the ones herein presented has been previously reported for other *S. rolfsii* strains (Deshpande *et al.* 1987; Kelkar *et al.* 1990). Nevertheless, comparing to the earlier protocols, the one currently proposed exhibits some advantages in terms of the required amount of enzyme, the length of digestion and the protoplast recovery. In this case, the lytic enzyme concentration could be almost twofold reduced and the incubation time was four times decreased when compared with the lowest reported values. With respect to the protoplast purification, the present one is much simpler as it does not involve layering or filtration steps.

Concluding, it was possible to standardize an efficient, simple, rapid and low-cost method for protoplast production in *Sclerotium rolfsii* ATCC 201126. In addition, the possibility to revert these protoplasts into the typical mycelial morphology was confirmed and attempts to increase regeneration rates are currently in progress. The usefulness of protoplast isolation for many different applications has been repeatedly mentioned and principally, if mutation or fusion procedures are thought as artificial improving methods, the multinucleated state of mycelium and the inability to produce conidia (a typical feature in the genus *Sclerotium*) might be overcome in this way by means of selecting uninucleated cells.

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REFERENCES

Chet, I. and Henis, Y. (1967) Chemical composition of hyphal and sclerotial walls of *Sclerotium rolfsii* Sacc. *Canadian Journal of Microbiology* **13**, 137–141.

Curragh, H.J., Mooibroek, H., Wessels, J.G.H., Marchant, R. and Mullan, E. (1992) Protoplast formation and DNA-mediated transformation of *Fusarium culmorum* to hygromycin B resistance. *Mycological Research* **97**, 313–317.

Deed, A. and Seviour, R. (1989) Formation and regeneration of protoplasts of *Sclerotium glucanicum*. *Applied Microbiology and Biotechnology* **31**, 259–264.

Deed, A. and Seviour, R.J. (1990) The application of fungal protoplasts in biotechnology. *Australian Journal of Biotechnology* **4**, 212–222.

Deshpande, M.V., Balkrishnan, H., Ranjekar, P.K. and Shankar, V. (1987) Isolation and immobilization of *Sclerotium rolfsii* protoplasts. *Biotechnology Letters* **9**, 49–52.

Fariña, J.I., Siñeriz, F., Molina, O.E. and Perotti, N.I. (1996a) Low-cost method for the preservation of *Sclerotium rolfsii* Proimi F-6656: inoculum standardization and its use in scleroglucan production. *Biotechnology Techniques* **10**, 705–708.

Fariña, J.I., Siñeriz, F., Molina, O.E. and Perotti, N.I. (1996b) Determination of radial growth rate of colonies of *Sclerotium rolfsii* F-6656 for the evaluation of culture medium, optimum incubation temperature, osmo- and halotolerance. *Revista Argentina de Microbiología* **28**, 190–196.

Fariña, J.I., Siñeriz, F., Molina, O.E. and Perotti, N.I. (1998) High scleroglucan production by *Sclerotium rolfsii*: influence of medium composition. *Biotechnology Letters* **20**, 825–831.

Fariña, J.I., Siñeriz, F., Molina, O.E. and Perotti, N.I. (2001) Isolation and physicochemical characterization of soluble scleroglucan from *Sclerotium rolfsii*. Rheological properties, molecular weight and conformational characteristics. *Carbohydrate Polymers* **44**, 41–50.

Gold, M.H., Cheng, T.M. and Alic, M. (1983) Formation, fusion, and regeneration of protoplasts from wild-type and auxotrophic strains of the white rot basidiomycete *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **46**, 260–263.

Hamlyn, P.F., Bradshaw, R.E., Mellon, F.M., Santiago, C.M., Wilson, J.M. and Peberdy, J.F. (1981) Efficient protoplast isolation from fungi using commercial enzymes. *Enzyme and Microbial Technology* **3**, 321–325.

Ishikawa, F. and Oishi, K. (1985) Formation and regeneration of protoplasts from *Conidiobolus lamprauges*. *Journal of General Microbiology* **131**, 3311–3316.

Kavanagh, K. and Whittaker, P.A. (1991) Effect of buffer viscosity on the rate of protoplast formation in yeasts. *Biomedical Letters* **46**, 57–62.

Kavanagh, K. and Whittaker, P.A. (1996) Application of protoplast fusion to the nonconventional yeast. *Enzyme and Microbial Technology* **18**, 45–51.

Kelkar, H.S., Shankar, V. and Deshpande, M.V. (1990) Rapid isolation and regeneration of *Sclerotium rolfsii* protoplasts and their potential application for starch hydrolysis. *Enzyme and Microbial Technology* **12**, 510–514.

Keller, U., Pöschmann, S., Krengel, U., Kleinkauf, H. and Kraepelin, G. (1983) Studies on protoplast fusion in *Streptomyces chrysomallus*. *Journal of General Microbiology* **129**, 1725–1731.

Kottutz, E. and Rapp, P. (1990) 1,3-β-Glucan synthase in cell-free extracts from mycelium and protoplasts of *Sclerotium glucanicum*. *Journal of General Microbiology* **136**, 1517–1523.

Magae, Y., Kakimoto, Y., Kashiwagi, Y. and Sasaki, T. (1985) Fruiting body formation from regenerated mycelium of *Pleurotus ostreatus* protoplasts. *Applied and Environmental Microbiology* **49**, 441–442.

Miller, J.C. and Miller, J.N. (1988) Significance tests. In *Statistics for Analytical Chemistry* ed. Chalmers, R.A. and Masson, M. pp. 53–80. Chichester: Ellis Horwood Ltd.

Okanishi, M., Suzuki, K. and Umezawa, H. (1974) Formation and reversion of streptomycete protoplasts: cultural condition and morphological study. *Journal of General Microbiology* **80**, 389–400.

Peberdy, J.F. (1980) Protoplast fusion – a tool for genetic manipulation and breeding in industrial microorganisms. *Enzyme and Microbial Technology* **2**, 23–29.

Picataggio, S.K., Schamhart, D.H.J., Montenecourt, B.S. and Eveleigh, D.E. (1983) Sphaeroplast formation and regeneration in *Trichoderma reesei*. *European Journal of Applied Microbiology and Biotechnology* **17**, 121–128.

Pretus, H.A., Ensley, H.E., McNamee, R.B., Jones, E.L., Browder, W. and Williams, D.L. (1991) Isolation, physicochemical characterization and preclinical efficacy evaluation of soluble scleroglucan. *Journal of Pharmacology and Experimental Therapeutics* **257**, 500–510.

Vázquez, F. and Figueroa, L.I.C. (1996) Protoplasts formation in *Fusarium* species. *Biotechnology Techniques* **10**, 93–98.

Yamada, O., Magae, Y., Kashiwagi, Y., Kakimoto, Y. and Sasaki, T. (1983) Preparation and regeneration of mycelial protoplasts of *Collybia veltipes* and *Pleurotus ostreatus*. *European Journal of Applied Microbiology and Biotechnology* **17**, 298–300.